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DNA taxonomy of Swedish Catenulida (Platyhelminthes) and a phylogenetic framework for catenulid classification

Karolina Larsson[a], Afsaneh Ahmadzadeh[a], Ulf Jondelius[b,*]

[a]*Systematic Biology, Evolutionary Biology Centre, Norbyvägen 18D, 752 36 Uppsala, Sweden*
[b]*Department of Invertebrate Zoology, Swedish Museum of Natural History, PO Box 50007, 104 05 Stockholm, Sweden*

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Abstract

Specimens of Catenulida were collected at 34 localities in Sweden. We used 18S rDNA, 28S rDNA, ITS-5.8S, and cytochrome oxidase I (COI) nucleotide sequences to infer phylogeny from parsimony jackknifing and Bayesian analysis. Our dataset contained 74 ingroup terminals and 5111 characters. The results show a basal split between a clade consisting of the marine Retronectidae + the limnic Catenulidae, and a second clade consisting of the limnic Stenostomidae. The hypothesis of the marine Retronectidae as the sister group of the limnic Catenulida is rejected. The recently introduced genus *Anokkostenostomum* Noreña, Damborenea & Brusa, 2005 results as non-monophyletic, and *Suomina* Marcus, 1945 as a group inside *Catenula* Dugès, 1832. Therefore, we propose to render *Anokkostenostomum* a new junior synonym of *Stenostomum* Schmidt, 1848, and *Suomina* a new junior synonym of *Catenula*. Consequently, the new combinations *Catenula evelinae* (Marcus, 1945), *Catenula sawayai* (Marcus, 1945), and *Catenula turgida* (Zacharias, 1902) are proposed, and 14 species are returned to their original genus, *Stenostomum*. The molecular phylogenetic hypothesis is used to identify and discriminate catenulid species. In our material, we found 12 species of Catenulida new to Sweden, and four species new to science, all of which are distinguishable by morphological characters.

Keywords: Catenulida; Platyhelminthes; Molecular phylogeny; DNA taxonomy

Introduction

Catenulida Meixner, 1924 is a group of small flatworms comprising about 100 species worldwide. Freshwater Catenulida, which constitute the vast majority of the species, live in mires, ponds, streams, and moist terrestrial habitats, where they often are very abundant. Marine Catenulida, on the other hand, are rare: only 12 species are known. Catenulids have a simple anatomy and lack sclerotized parts, such as the copulatory stylets common in, e.g., rhabdocoel flatworms. Many characters (e.g. shape, size, colour) show high intraspecific variability, which makes species identification problematic. Catenulids, which are very fragile, can be identified only when alive, and are rarely encountered in a sexually mature stage as they normally reproduce by paratomy. Many currently recognized catenulid species are regarded as cosmopolites

*Corresponding author.
E-mail address: ulf.jondelius@nrm.se (U. Jondelius).

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(e.g. *Stenostomum leucops* Dugès, 1828; *Catenula lemnae* Dugès, 1832). There is, however, a potential for cryptic diversity among the cosmopolitan morphological species, due to the paucity of distinguishing features.

The Swedish catenulid fauna is virtually unknown, with only two limnic species, *Stenostomum karlingi* (Luther 1960), and *S. leucops*, reported by Luther (1960). Sterrer and Rieger (1974) recorded three marine species: *Retronectes clio* (Sterrer and Rieger 1974), *R. melpomene* (Sterrer and Rieger 1974), and one *Retronectes* sp., all found in very low numbers. However, our studies have revealed that limnic catenulids are highly abundant in Sweden. But how many species are there? Here we aim to provide a phylogenetic framework for the Catenulida and to sample predominantly limnic catenulids from various habitats in Sweden in order to establish the number of Swedish species and their identity.

Ideally, a phylogenetic study of the Catenulida should encompass specimens collected worldwide. Such material was not available to us, but even though the specimens sequenced by us had been collected exclusively within Sweden, the dataset used in the analyses, which was complemented by the catenulid sequences available in GenBank, included material from three of the five families, and six of the 12 catenulid genera currently recognized. We were not able to collect any of the five species of the Chordariidae Marcus, 1945, nor the single species in Tyrrheniellidae Riedl, 1959.

In this first attempt to use molecular data to reconstruct the phylogeny of the Catenulida and to provide a framework for a phylogenetic classification, we used the mitochondrial cytochrome oxidase I (COI) gene and three ribosomal markers: the 18S rDNA gene, the 28S rDNA gene, and the ITS1-5.8S-ITS2 rDNA region (ITS-5.8S). Our aim was also to identify cryptic diversity at the species level among these microscopic worms. The use of four common molecular markers provided an opportunity to evaluate their performance in identifying species-level taxa. We then tried to identify morphological characters for the species groups, in order to be able to distinguish the latter based on morphology at the time of collection. Formal taxonomic descriptions of the new species we have identified will be the subject of a separate study (Larsson and Willems, unpublished), which will also include detailed anatomical data. In the present paper, those new species are referred to with provisional names based on their morphological characteristics.

The phylogenetic hypothesis based on our combined data permitted us to examine some evolutionary problems within the Catenulida, such as the proposed sister-group relationship between marine and limnic catenulids (Ehlers 1994). Our choice of a parsimony-based method to identify cryptic diversity has an important advantage over distance-based methods used in some barcoding studies (e.g. Hebert et al. 2003), since it generates character-based hypotheses of evolutionary relationships also in cases where species delimitation is ambiguous (Will and Rubinoff 2004). DNA-barcoding studies have focused on the identification of known species, but a greater challenge lies in the application of DNA-based methods to poorly characterized taxa (Monaghan et al. 2005) such as the Catenulida.

Material and methods

Collection and identification of specimens

Catenulids were sampled during 2003–2005 from 34 locations in Sweden (Table 1). The specimens were collected by searching samples of moss, other vegetation or sediment under a stereomicroscope, and then identified live under a microscope. Live worms were

Table 1. Sampling data

Locality	Province	Date (year-month-day)
01	Bohuslän	03-07-26, 04-07-15
02	Uppland	04-05-06
03	Småland	04-05-15
04	Jämtland	04-06-30
05	Jämtland	04-06-30
06	Jämtland	04-07-03
07	Jämtland	04-07-03, 05-06-23
08	Jämtland	04-07-06
09	Bohuslän	04-07-22, 05-07-27
10	Bohuslän	04-07-28
11	Bohuslän	04-07-27
12	Bohuslän	04-08-02
13	Bohuslän	04-08-03
14	Bohuslän	04-08-04
15	Öland	04-09-09
16	Småland	04-09-14
17	Småland	04-09-14
18	Gotland	04-09-18
19	Gotland	04-09-20
20	Gotland	04-09-20
21	Gotland	04-09-20
22	Skåne	04-10-05
23	Jämtland	05-06-23
24	Jämtland	05-06-24
25	Jämtland	05-06-24
26	Jämtland	05-06-25
27	Jämtland	05-06-25
28	Jämtland	05-06-30
29	Jämtland	05-06-30
30	Lappland	05-07-01
31	Lappland	05-07-08
32	Bohuslän	05-08-01
33	Bohuslän	05-08-06
34	Jämtland	05-06-23

photographed and drawings were made before specimens were preserved in 95% ethanol. Material for histological study was preserved in Bouin's fluid.

DNA extraction, PCR amplification and sequencing

DNA was extracted from 74 ethanol-preserved specimens using the DNeasy Tissue Kit (Qiagen), following the manufacturer's protocol. Amplifications were performed with 2 µl DNA extract and 1 µl of each primer using Ready-To-Go PCR beads (Amersham Biosciences), each containing 2.5 U of PuReTaq DNA Polymerase, 10 mM Tris–HCl, 50 mM KCl, and 1.5 mM $MgCl_2$, 200 µM each of dNTP and stabilizers including bovine serum albumin. The final volume was 25 µl. 18S rDNA was amplified in two overlapping fragments using the primer combination 4fb + 1806R (1200 base pairs) and 5fk + S30 (900 base pairs). 28S rDNA was amplified with the primers LSU5 + L1642R (1450 base pairs), COI with the primers COI3B + COI5B (600 base pairs), and ITS-5.8S was amplified in two fragments with the primers ITS4 + ITS5 (950 base pairs) (for primer sequences and references, see Table 2). Products were purified with the QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's protocol. The PCR products were sequenced by Macrogene Inc. (Seoul, Korea), using the additional internal primers listed in Table 2. Sequences were assembled and edited using the software STADEN (Judge et al. 2001).

Due to shortage of material and problems with PCR amplification in some species, overlap among datasets is incomplete. We were able to sequence 18S rDNA from 54 specimens of catenulids, 28S rDNA from 47 specimens, COI from 42 specimens, and ITS-5.8S from 54 specimens. The sequences are listed in Table 3, the additional sequences downloaded from GenBank in Table 4. The four genes were aligned separately using the software MUSCLE (Edgar 2004). Hypervariable regions were manually deleted from the resulting alignment. The final lengths of the datasets were 1803 bp (18S), 1553 bp (28S), 599 bp (COI), and 1156 bp (ITS-5.8S), respectively.

Phylogenetic reconstruction

Parsimony jackknifing (Farris et al. 1996) was performed on the combined 18S rDNA + 28S rDNA + COI + ITS-5.8S dataset, using the software TNT (Goloboff et al. 2003). We performed 1000 jackknife replicates each, with 50 random additions, TBR branch swapping and a deletion frequency of 36%. The results from parsimony jackknifing were summarized in a majority-rule tree (Fig. 1) with the cut-off value at 70%. We also performed parsimony-jackknife analyses on the separate gene datasets, using TNT with the same parameter settings as above (Figs. 2 and 3). Bremer support (BS) values (Bremer 1988) were calculated on the strict consensus tree from the parsimony analysis with the combined dataset, using PAUP* 4b.10 (Swofford 2002). We used the software MrModeltest (Nylander 2004) on the combined data to decide which model of sequence evolution to use for a Bayesian phylogenetic analysis. The latter was performed with MrBayes v3.1.2 (Ronquist and Huelsenbeck 2003), using a GTR + Γ model with four MCMC chains running for 12,380,000 generations sampled every 500 trees after a burn-in of 13,800 trees. The results were summarized in a majority-rule consensus tree (Fig. 1, right-side tree showing branch lengths and Bayesian posterior probabilities (BPP)).

Table 2. Primers used for amplification and sequencing

Primer	Gene	Used for	Primer sequence (5′–3′)	Reference
S30	18S	PCR	GCT TGT CTC AAA GAT TAA GCC	Norén and Jondelius (1999)
5fk	18S	PCR	TTC TTG GCA AAT GCT TTC GC	Norén and Jondelius (1999)
4fb	18S	PCR	CCA GCA GCC GCG GTA ATT CCA G	Norén and Jondelius (1999)
1806R	18S	PCR	CCT TGT TAC GAC TTT TAC TTC CTC	Norén and Jondelius (1999)
7fk	18S	Seq.	GCA TCA CAG ACC TGT TAT TGC	Norén and Jondelius (1999)
4fbk	18S	Seq.	CTG GAA TTA CCG CGG CTG CTG G	Norén and Jondelius (1999)
7f	18S	Seq.	GCA ATA ACA GGT CTG TGA TGC	Norén and Jondelius (1999)
5f	18S	Seq.	GCG AAA GCA TTT GCC AAG AA	Norén and Jondelius (1999)
L300F	28S	PCR, seq.	CAA GTA CCG TGA GGG AAA GTT G	Littlewood et al. (2000)
LSU5	28S	PCR, seq.	TAG GTC GAC CCG CTG AAY TTA AGC A	Littlewood et al. (2000)
L1642R	28S	PCR, seq.	CCA GCG CCA TCC ATT TTC A	Lockyer et al. (2003)
COI3B	COI	PCR, seq.	AAG TGT TGN GGR AAR AAN GT	Telford et al. (2000)
COI5B	COI	PCR, seq.	TTC TGR TTY TTY GGN CAY CC	Telford et al. (2000)
ITS4	ITS-5.8S	PCR, seq.	TCC TCC GCT TAT TGA TAT GC	White et al. (1990)
ITS5	ITS-5.8S	PCR, seq.	CGA AGT AAA AGT CGT AAC AAG G	White et al. (1990)

Table 3. Data on specimens and sequences obtained

Specimen	Taxon	Locality	18S rDNA	28S rDNA	ITS-5.8S	COI
K03:13	*Catenula lemnae*	01	FJ196318	FJ196318	–	–
K04:01	*Stenostomum sphagnetorum*	02	FJ384797	–	–	FJ384873
K04:03	*Anokkostenostomum bryophilum*	03	FJ196319	–	FJ384911	–
K04:04	*Anokkostenostomum bryophilum*	03	–	FJ384835	FJ384912	–
K04:06	*Anokkostenostomum grabbskogense*	04	FJ196326	FJ196337	FJ384913	FJ384874
K04:08	*Suomina turgida* 1	04	FJ384798	–	FJ384914	–
K04:09	*Anokkostenostomum bryophilum*	05	FJ384799	FJ384836	FJ384915	FJ384875
K04:11	*Anokkostenostomum grabbskogense*	06	FJ196327	FJ196338	FJ384917	FJ384876
K04:12	*Anokkostenostomum grabbskogense*	06	–	FJ384838	FJ384918	FJ384877
K04:15	*Anokkostenostomum grabbskogense*	05	–	FJ384839	–	FJ384878
K04:18	*Stenostomum leucops*	06	FJ384800	FJ384840	FJ384919	FJ384879
K04:19	*Anokkostenostomum grabbskogense*	06	FJ384801	FJ384841	FJ384920	FJ384880
K04:22	*Suomina turgida* 1	08	FJ384802	–	FJ384921	–
K04:28	*Suomina turgida* 2	01	FJ384803	–	FJ384922	–
K04:29	*Stenostomum leucops*	01	FJ384804	FJ384842	FJ384923	FJ384881
K04:30	*Anokkostenostomum bryophilum*	01	FJ196320	FJ384843	FJ384924	FJ384882
K04:32	*Suomina turgida* 1	08	FJ384805	FJ196339	FJ384925	FJ384883
K04:40	*Rhynchoscolex simplex*	01	FJ196328	–	FJ384926	FJ384884
K04:41	*Rhynchoscolex simplex*	01	FJ384806	FJ384844	FJ384927	FJ384885
K04:43	*Suomina turgida* 2	10	FJ196329	–	FJ384928	FJ384886
K04:45	'*Anokkostenostomum* bigmouth'	09	FJ196330	FJ196341	FJ384929	FJ384887
K04:49	*Anokkostenostomum bryophilum*	01	–	FJ384845	FJ384930	FJ384888
K04:50	*Anokkostenostomum bryophilum*	01	FJ384807	FJ384846	–	–
K04:53	*Stenostomum arevaloi*	09	FJ384808	FJ384847	FJ384931	FJ384889
K04:59	'*Anokkostenostomum* smallpit'	01	FJ196331	–	–	FJ384890
K04:63	*Stenostomum leucops*	11	FJ196332	FJ196342	FJ384932	FJ384891
K04:67	*Catenula macrura*	12	FJ196321	–	FJ384933	–
K04:69	*Catenula lemnae*	13	FJ196322	–	FJ384934	–
K04:71	*Anokkostenostomum bryophilum*	12	FJ196333	FJ196343	FJ384935	FJ384892
K04:75	*Stenostomum leucops*	10	FJ384809	FJ384848	FJ384936	FJ384893
K04:78	*Anokkostenostomum bryophilum*	08	FJ196334	FJ196344	FJ384937	FJ384894
K04:80	*Stenostomum arevaloi*	14	FJ384810	FJ384849	FJ384938	FJ384895
K04:81	'*Stenostomum* island'	15	FJ384811	FJ384850	–	FJ384896
K04:84	'*Anokkostenostomum* smallpit'	16	–	FJ384851	FJ384939	FJ384897
K04:85	*Stenostomum leucops*	17	FJ384812	FJ384852	FJ384940	FJ384898
K04:87	*Stenostomum leucops*	16	FJ384813	FJ384853	FJ384941	FJ384899
K04:88	*Stenostomum leucops*	18	FJ384814	FJ384854	FJ384942	–
K04:90	'*Stenostomum* island'	19	–	FJ384855	–	FJ384900
K04:93	'*Stenostomum* island'	21	–	FJ384856	FJ384943	FJ384901
K04:94	'*Stenostomum* island'	20	–	–	FJ384944	FJ384902
K04:102	*Catenula lemnae*	22	FJ196324	–	–	–
K04:104	*Stenostomum sphagnetorum*	22	–	FJ384837	–	
K04:109	*Catenula lemnae*	22	FJ196325	–	FJ384916	–
K05:01	'*Anokkostenostomum* longpit'	23	FJ384815	FJ384857	FJ384945	–
K05:02	'*Anokkostenostomum* longpit'	07	FJ384816	FJ384858	FJ384946	–
K05:04	*Rhynchoscolex simplex*	24	FJ384817	FJ384859	–	FJ384903
K05:07	*Stenostomum sphagnetorum*	25	FJ384818	FJ384860	–	FJ384904
K05:10	*Catenula lemnae*	27	FJ384819	–	FJ384947	–
K05:12	*Anokkostenostomum grabbskogense*	26	FJ384820	FJ384861	FJ384948	–
K05:14	'*Anokkostenostomum* mountain'	34	FJ384821	FJ384862	FJ384949	–
K05:15	*Anokkostenostomum grabbskogense*	26	FJ384822	FJ384863	FJ384950	–
K05:17	'*Anokkostenostomum* longpit'	23	FJ384823	FJ384864	FJ384951	FJ384905
K05:20	'*Anokkostenostomum* longpit'	23	FJ384824	FJ384865	FJ384952	FJ384906
K05:24	*Suomina turgida* 1	28	FJ384825	–	FJ384953	–
K05:26	*Stenostomum leucops*	29	FJ384826	FJ384866	FJ384954	–

Table 3. (*continued*)

Specimen	Taxon	Locality	18S rDNA	28S rDNA	ITS-5.8S	COI
K05:27	*Anokkostenostomum grabbskogense*	29	FJ384827	FJ384867	FJ384955	FJ384907
K05:37	*Stenostomum leucops*	31	FJ384828	FJ384868	FJ384956	–
K05:50	*Catenula lemnae*	32	FJ384829	–	FJ384957	–
K05:51	*Stenostomum leucops*	32	FJ384830	FJ384869	FJ384958	FJ384908
K05:52	'*Anokkostenostomum* bigmouth'	33	FJ384831	–	FJ384959	–
K05:55	*Stenostomum leucops*	33	FJ384832	FJ384870	FJ384960	FJ384909
K05:60	*Stenostomum arevaloi*	33	FJ384833	FJ384871	FJ384961	FJ384910
K05:61	*Catenula lemnae*	33	FJ384834	–	FJ384962	–
K05:64	'*Anokkostenostomum* smallpit'	33	–	FJ384872	–	–

Results

Combined dataset

There is a primary dichotomy between the Catenulidae–Retronectidae clade and the Stenostomidae clade, which both receive maximum parsimony-jackknife support and Bayesian Posterior probability (Fig. 1). Within the first clade Catenulidae and Retronectidae are sister taxa, with maximal support indices. *Rhynchoscolex* Leidy, 1851 is the sister group of the other terminals in the Stenostomidae clade, also with maximum support. The sister group of *Rhynchoscolex* is a taxon consisting of the terminals *Stenostomum* (Schmidt 1848) and *Anokkostenostomum* Noreña, Damborenea & Brusa, 2005. Resolution within this clade is incomplete, but the terminals assigned to *Anokkostenostomum* do not form a monophylum.

Inferred branch lengths in the majority-rule consensus tree summarizing the Bayesian analyses are shorter within the Stenostomidae clade, longer in Catenulidae–Retronectidae.

Clades consisting exclusively of terminals that could be assigned to a single named species and clades consisting exclusively of terminals that could not be assigned to any named species based on morphology were considered to represent distinct species if they received maximum parsimony-jackknife support, and BS greater than 10. There are 14 such clades in our combined dataset. Nine of these groups also had a BPP of 1.0. Within these 14 groups there is internal structure, with lower support in six (parsimony) and 11 (Bayesian) of them, respectively. This may indicate the existence of additional species, but as the sampling was limited in geographical coverage and number of specimens for many of our taxa, we here discern only species that we

Table 4. GenBank sequences used, and corresponding taxa

Higher taxon	Species	18S rDNA	28S rDNA	ITS-5.8S	COI
Annelida	*Procerea cornuta*	AF212179	AF212165	AF21265	
	Eucyllis blomstrandi				AY839579
Catenulida	*Paracatenula* cf. *erato*	AY218103	AY218130		
	Paracatenula cf. *polyhymnia*	AY218104	AY218131		
	Stenostomum leucops	U70084	AY157151		
	Suomina sp.	AJ012532	AF021322		
Macrostomida	*Microstomum lineare*	U70082	AJ270172		AJ405980
Mollusca	*Lottia digitalis*	DQ248942	DQ248942	DQ248942	DQ238599
Polycladida	*Notoplana australis*				AJ405981
	Stylochus zebra	AF342801	AF342800		
Rhabdocoela	*Mesostoma lingua*	AJ270157	AJ270171		AJ405988
Tricladida	*Bdelloura candida*		AY157154		
	Dugesia japonica	M58344			D49916
	Schmidtea mediterranea			AF047854	

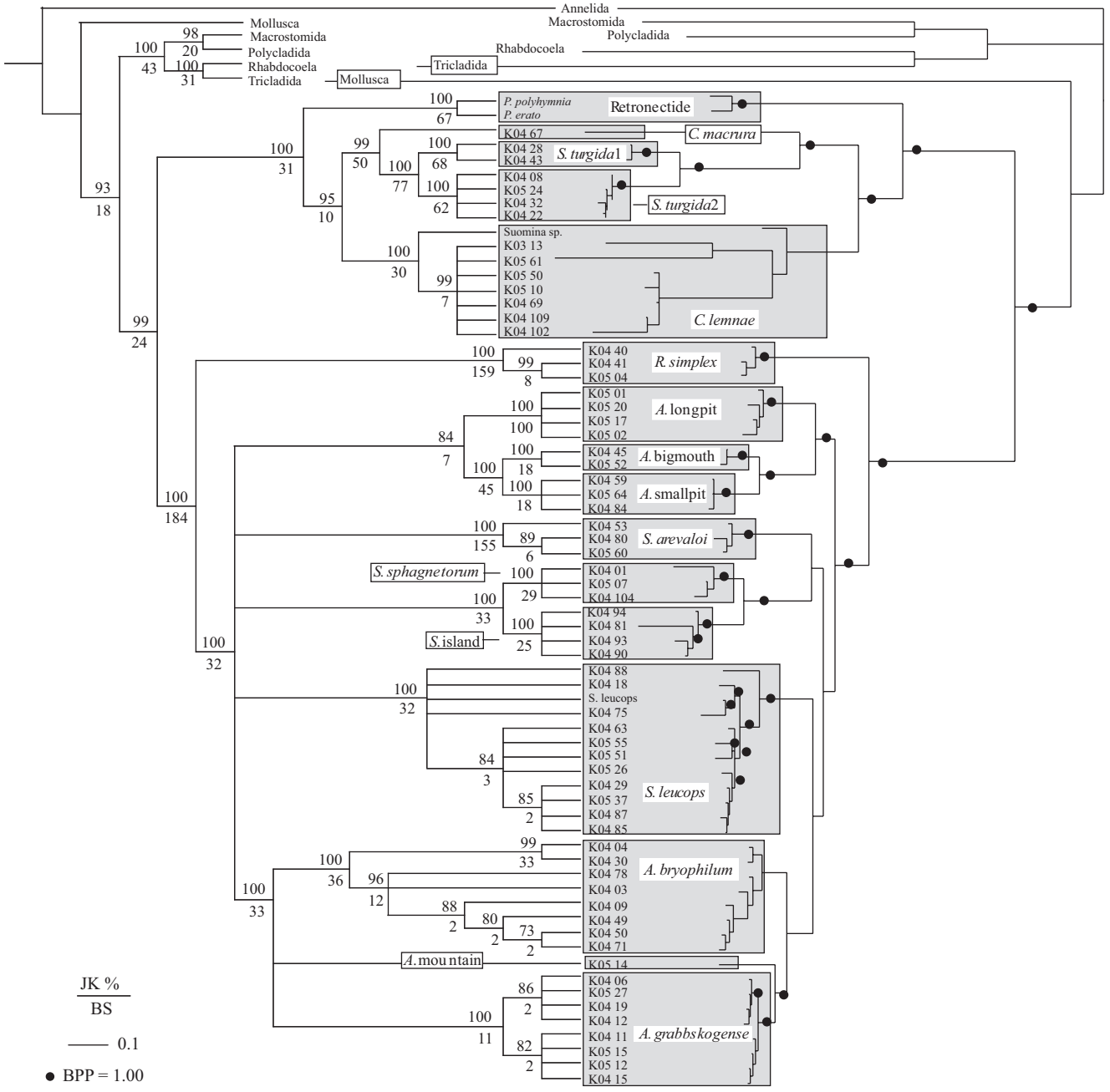

Fig. 1. Phylogeny of Catenulida as reconstructed from the combined dataset of 18S, 28S, COI, and ITS-5.8S sequences; species groups shown as grey boxes. Left-side tree: majority-rule consensus with cut-off value at 70% from jackknife parsimony analysis; jackknife frequencies shown above branches, Bremer support below branches. Right-side tree: majority-rule consensus with branchlengths from Bayesian analysis; black circle indicates BPP of 1.00.

can also identify based on morphological characters (Fig. 4). The status of the remaining clade, consisting of a single terminal that could not be assigned to a nominal species, cannot be determined.

The 12 clades identified as named species are (Fig. 1 and Table 5): *Anokkostenostomum bryophilum* (Luther 1960); *A. grabbskogense* (Luther 1960); *Catenula macrura* Marcus, 1945; *C. lemnae* Dugès, 1832; *Rhynchoscolex simplex* Leidy, 1851; *Stenostomum arevaloi* Gieysztor, 1931; *S. sphagnetorum* (Luther 1960); *S. leucops* (Dugès, 1828); *Suomina turgida* (Zacharias, 1902) (this name being assigned to two groups); and *Paracatenula erato* (Sterrer and Rieger 1974) and *P. polyhymnia* (Sterrer and Rieger 1974) (the latter two based on publicly available sequences; see Table 4). Four of the species-level clades could not be assigned to any described species, thus are regarded as new species. For three of these there are also distinguishing morphological features

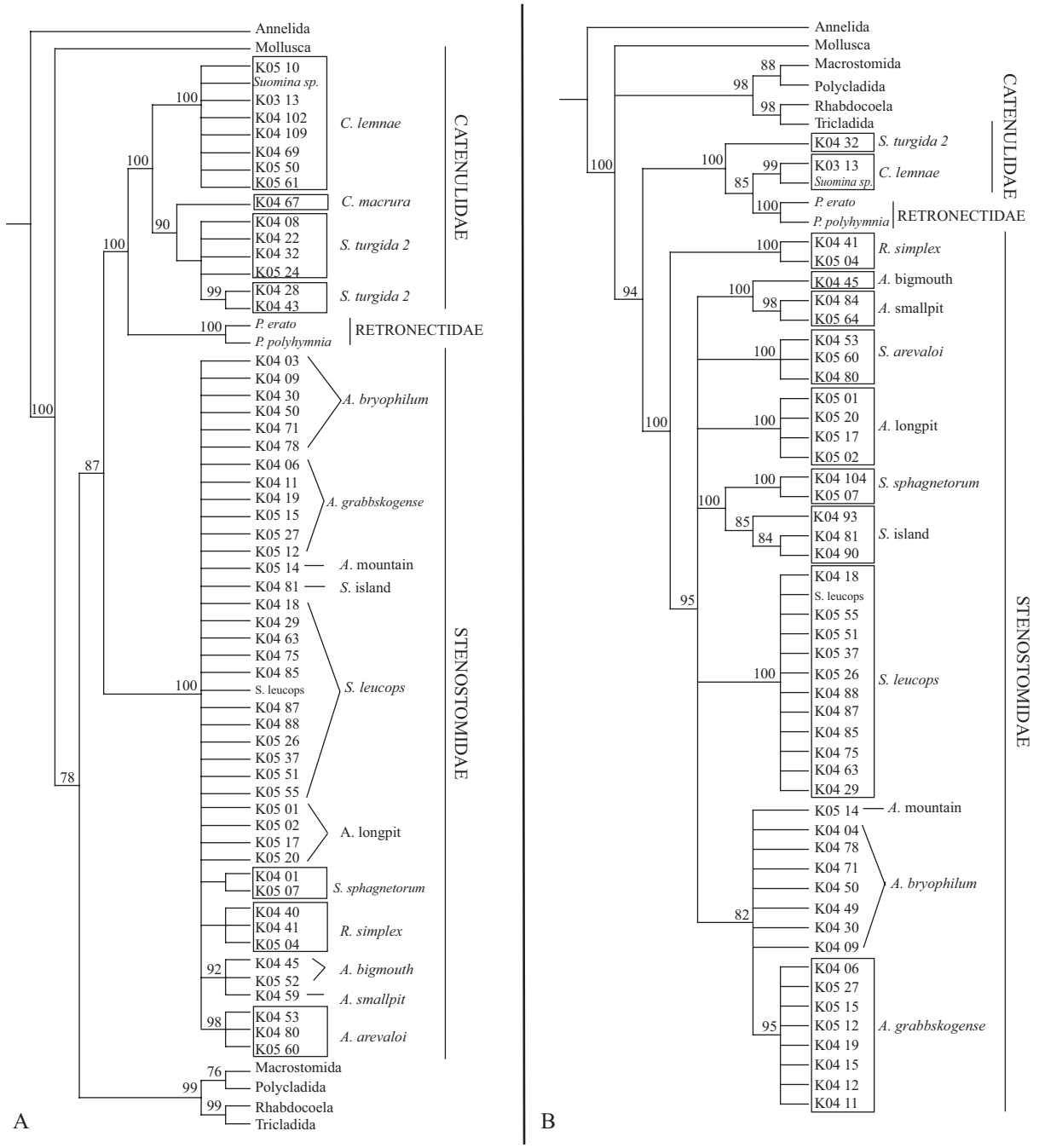

Fig. 2. Phylogeny of Catenulida as reconstructed from jackknife parsimony analyses of the 18S (A) and 28S (B) datasets, respectively; jackknife frequencies shown above branches.

(see below). One of the species groups did not have any uniformly assigned distinguishing morphological character; however, the four specimens in this group were found exclusively on the Baltic islands Gotland and Öland.

Single-marker datasets

As noted above, overlap between the single-gene datasets was incomplete due to difficulties with PCR amplification and shortage of template DNA for some of our terminals (Table 3). The markers, nuclear and mitochondrial, support congruent groups but with different degrees of resolution. There are nine species-level monophyletic groups in our 18S rDNA jackknife tree (Fig. 2A). A basal split between the Catenulidae–Retronectidae clade and the Stenostomidae clade is present. Within the Catenulidae the deeper branches are resolved, whereas resolution is low in the Stenostomidae clade, with only three species groups supported. The 28S

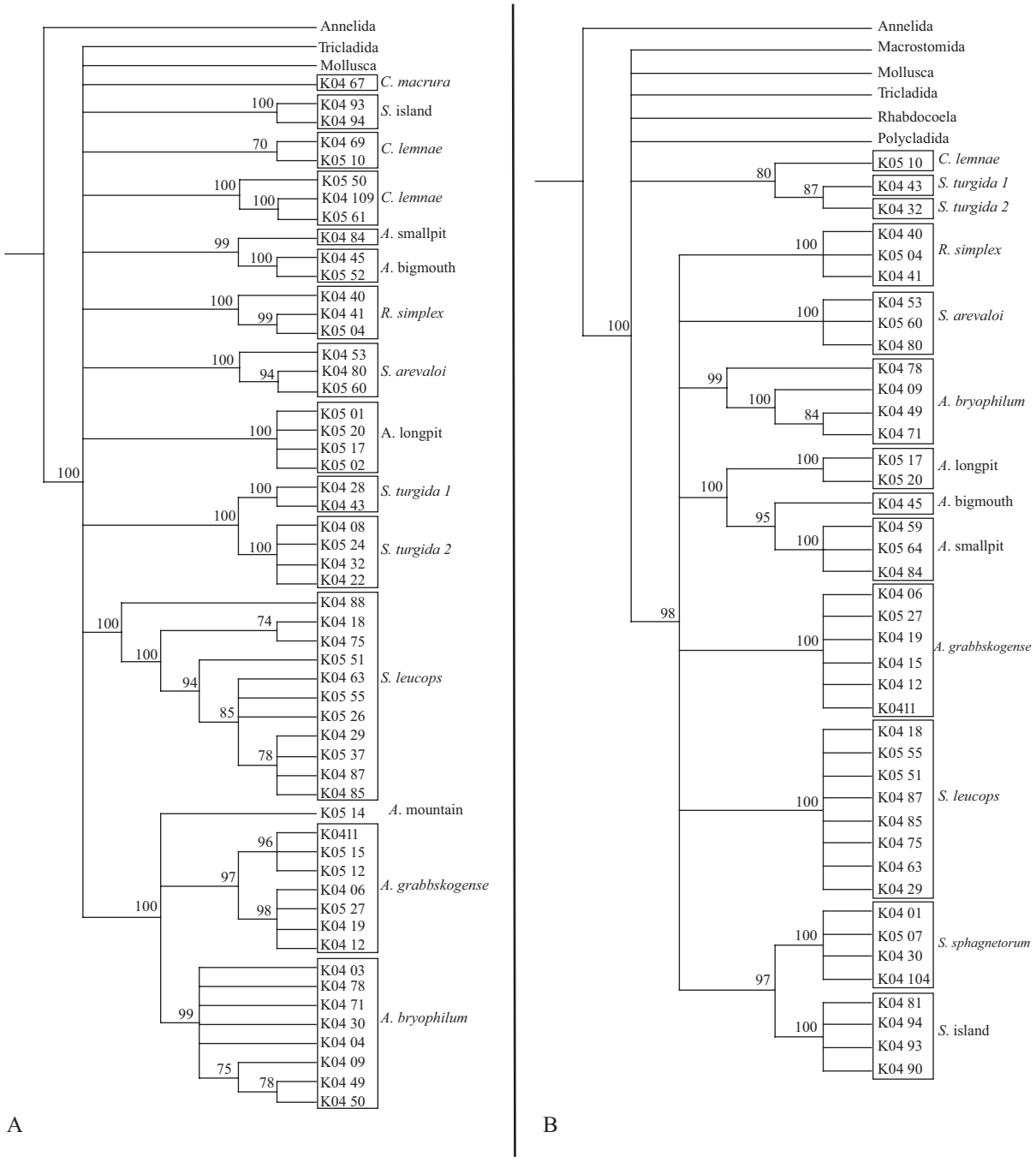

Fig. 3. Phylogeny of Catenulida as reconstructed from jackknife parsimony analyses of the ITS-5.8S (A) and COI (B) datasets, respectively; jackknife frequencies shown above branches.

rDNA dataset (Fig. 2B) resolves 13 species-level groups, including nine species within Stenostomidae. The basal split between the Catenulidae–Retronectidae clade and the Stenostomidae clade is also recovered. The ITS-5.8S data partition (Fig. 3A) does not support deeper nodes: the Catenulidae–Retronectidae and the Stenostomidae clades as well as the split between *Rhyncoscolex* and *Stenostomum* + *Anokkostenostomum* are not present. However, 15 species groups are present in this dataset. The ITS-5.8S is the only data partition that reveals strongly supported structure within the nominal species *S. leucops*. The mitochondrial COI (Fig. 3B) weakly supports the Catenulidae clade (no data for Retronectidae), and jackknife support is strong for the Stenostomidae clade. Thirteen species groups are supported.

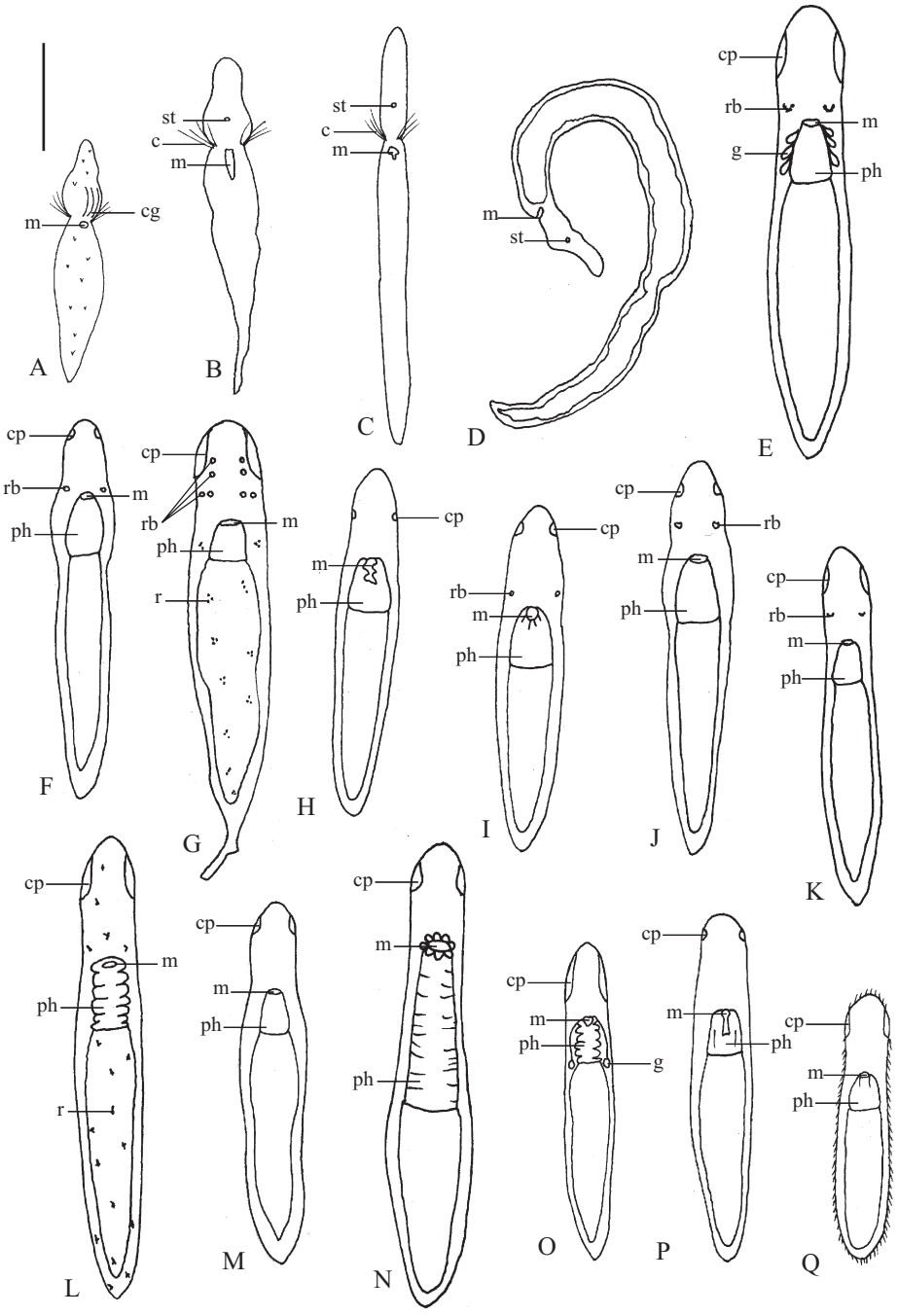

Fig. 4. Freehand drawings of representatives of the catenulid species groups found; distinguishing morphological characters indicated: (A) *Suomina turgida*, (B) *Catenula macrura*, (C) *Catenula lemnae*, (D) *Rhynchoscolex simplex*, (E) *Stenostomum leucops*, (F) *Stenostomum sphagnetorum*, and (G) *Stenostomum arevaloi*. (H–K) '*Stenostomum* island', (L) *Anokkostenostomum bryophilum*, (M) *Anokkostenostomum grabbskogense*, (N) '*Anokkostenostomum* bigmouth', (O) '*Anokkostenostomum* longpit', (P) '*Anokkostenostomum* smallpit', and (Q) '*Anokkostenostomum* mountain'. Abbrevations: c = cilia, cg = ciliated girdle, cp = ciliated pits, g = pharyngeal gland, m = mouth, ph = pharynx, r = epidermal inclusion, rb = refractile body, st = statocyst. Scale bar = 0.25 mm.

Discussion

Our analysis of the combined dataset yielded a strongly supported phylogenetic hypothesis. The basal split between the Catenulidae–Retronectidae and the Stenostomidae clades is incompatible with Ehlers' (1994) system for the Catenulida, in which Retronectidae was the sister group of Catenulidae + Stenostomidae. Ehlers' hypothesis was based on the ultrastructure of the protonephridium. The synapomorphy proposed for the latter

two groups (Ehlers 1994) is the presence of transverse or zigzag bars and horizontal clefts in the filter area of the protonephridium; longitudinal clefts occur in Retronectidae, a condition that also occurs in, e.g., Gnathostomulida (Ehlers 1994). Under our hypothesis the transverse bars and horizontal clefts are independently acquired in Stenostomidae and Catenulidae. Within the Catenulida, statocysts occur in Retronectidae, Catenulidae, and *Rhyncoscolex*. We propose loss of the statocyst as a synapomorphy for the *Stenostomum* clade.

Species groups

The eight specimens we identified as *Anokkostenostomum bryophilum* (Fig. 4L) form a group that is supported in the analyses of the combined dataset and of ITS-5.8S and COI, respectively. The morphological character uniting the specimens is epidermal inclusions that occur singly or in duets. There is some hierarchical structure among the eight terminals in the combined analyses derived from the ITS-5.8S and COI datasets, although there is limited resolution. Our specimens were all collected in streams and pools near Storulvån in the province of Jämtland. We regard them all as *A. bryophilum*.

Anokkostenostomum grabbskogense (Fig. 4M) forms a group of eight terminals that is well supported in the analyses of the combined data and of the single-marker partitions except 18S rDNA. Two internal groups are supported in the analyses of the combined dataset and the ITS-5.8S partition. Inferred branch lengths within *A. grabbskogense* in the Bayesian analysis of the combined data are very short (much shorter than in *S. leucops*). Our specimens were collected at different localities in the province of Jämtland. We regard the divergence between our *A. grabbskogense* specimens as variation at the population level.

The *Catenula lemnae* (Fig. 4C) clade consists of seven terminals collected by us at different locations in Sweden; in addition there is one terminal identified as *Suomina* sp. that is represented by GenBank sequences (AJ012532, AF021322). This *Suomina* sp. combines with our *C. lemnae* sequences to form a strongly supported group: jackknife support and the BPP are maximal for this group, and the BS value is 30. *Suomina* sp. does not group with our own *Suomina* terminals, but is separated from them by two strongly supported nodes. Thus, *Suomina* sp. (AJ012532, AF021322) could be a misidentification, or *Catenula* and *Suomina* are not monophyletic.

Catenula macrura (Fig. 4B) is represented by a single terminal. It is morphologically distinct from *C. lemnae* and *Suomina*. *Catenula macrura* is the sister group to *Suomina turgida* in our combined tree; this is strongly supported both by parsimony and the Bayesian analysis, and also by the 18S rDNA single-gene analysis. This warrants transfer of *S. turgida* to *Catenula*.

Rhynchoscolex simplex (Fig. 4D) is a large catenulid and very distinct morphologically. It is unambiguously placed as the sister group of the *Stenostomum* clade. The three specimens representing *R. simplex* form a monophylum, but with internal structure, as two of the terminals form a strongly supported group in the combined tree. Support for this subdivision of *R. simplex* is derived from the ITS-5.8S data partition, which also detected phylogenetic structure within the nominal species *Stenostomum leucops* (see below). However, our material is very limited, there are no morphological differences between the specimens, and the inferred branch lengths in the Bayesian analysis are short. Therefore we regard our *Rhynchoscolex* specimens as representing a single species.

The *Stenostomum arevaloi* group (Fig. 4G) consists of three specimens. The morphology of this species is distinct: the shape of the three pairs of refractile bodies, the tail, and the epidermal inclusions that occur in clusters are all characteristic. Two of the three *S. arevaloi* terminals form a moderately supported group in the combined analysis (jackknife frequency 89%). Again this grouping is supported mainly by the ITS-5.8S data partition (94% jackknife frequency). In view of the limited number of specimens, short inferred branch lengths in the Bayesian analysis, and the lack of morphological characters separating the *S. arevaloi* terminals, we consider our specimens as conspecific.

The *Stenostomum sphagnetorum* group (Fig. 4F), with three specimens, is well supported in the analyses of the combined data and in the 28S rDNA and COI analyses; it is monophyletic in the 18S partition analysis. The spherical shape of the refractile bodies with a central indentation is characteristic, as well as the shape of the anterior part of the body which is very similar to Luther's (1960, Fig. 7) description.

Stenostomum leucops (Fig. 4E) is a widely distributed and frequently encountered catenulid with highly variable size and pigmentation. It is characterized by the refractile bodies being composed of several small granules. Its large distribution and morphological variability suggest that it is a species complex. We found *S. leucops* in many localities in Sweden. In our analyses, the *S. leucops* group, which consists of 12 specimens, is strongly supported by the combined dataset and all single-gene datasets except the 18S rDNA partition. *Stenostomum leucops* is relatively well sampled, and in the Bayesian analysis of the combined data there are six internal clades with maximum BPP. The internal structure is again derived primarily from the ITS-5.8S data partition, which resolves five internal groups with jackknife support between 74% and 100%. *Stenostomum leucops* remains a candidate for species-complex status, but we have not been able to find any

correlation between the internal groups and morphology or distribution. Inferred branch lengths within *S. leucops* range from very short to longer than the branches separating *A. bryophilum* and *A. grabbskogense* in the combined analysis. This may be interpreted as evidence for ongoing cladogenesis in *S. leucops*, some populations of which may be regarded as separate species.

Our six specimens representing *Suomina turgida* (Fig. 4A) were all collected at localities around Sweden. Initially we identified the specimens as *S. turgida*, but there are two distinct clades (*S. turgida* 1 and *S. turgida* 2 in Figs. 1–3) in our combined tree, both with maximum jackknife support and BS above 60. The two groups are also supported by 18S and ITS-5.8S data in the single-gene analyses. *S. turgida* 1 was collected in Jämtland province; *S. turgida* 2 at localities in Bohuslän province. Hence we might conclude that there are two species of *Suomina* in our dataset. However, since we observed no morphological differences between them, *S. turgida* may consist of two cryptic species, but our material is too limited to draw any firm conclusions.

New species

'*Anokkostenostomum* bigmouth' (Fig. 4N) has a large, muscular pharynx and a normal, non-muscular intestine; the large pharynx is somewhat similar to the condition in *Myostenostomum*. There are considerable diffferences, however, as the five species of *Myostenostomum* are characterized by a muscular anterior part of the intestine in addition to a muscular pharynx (Luther 1960). Our two specimens form a well-supported group in the analysis of the combined dataset and also in the ITS-5.8S analysis. We regard this as a new species.

The three specimens that were identified as an undescribed species provisionally referred to as '*Anokkostenostomum* smallpit' (Fig. 4P) form a strongly

Table 5. Species groups inferred from phylogenetic reconstruction, corresponding specimens, and genetic, morphological and distributional support for the respective clade (jk = jackknife frequency)

Species group	Specimens	Genes (jk > 70%)	Morphology; distribution
'*Anokkostenostomum* bigmouth'	K04:45, K05:52	ITS	Very large pharynx
Anokkostenostomum bryophilum	K04:03, K04:78, K04:09, K04:49, K04:50, K04:71, K04:04, K04:30	ITS, COI	Epidermal inclusions
Anokkostenostomum grabbskogense	K04:11, K04:15, K05:12, K05:15, K04:12, K04:19, K05:27, K04:06	ITS, 28S, COI	Wrinkled pharynx; found only in Jämtland
'*Anokkostenostomum* longpit'	K05:01, K05:02, K05:17, K05:20	ITS, 28S, COI	Long ciliated pits
'*Anokkostenostomum* mountain'	K05:14	–	Dense ciliation
'*Anokkostenostomum* smallpit'	K04:59, K04:84, K05:64	28S, COI	Short ciliated pits
Catenula lemnae + *Suomina* sp. (GenBank)	K03:13, K04:69, K04:102, K04:109, K05:10, K05:50, K05:61; GenBank	28S, 18S	Statocyst
Catenula macrura	K04:67	–	Long tail, statocyst
Paracatenula erato	GenBank	28S, 18S	
Paracatenula polyhymnia	GenBank	28S, 18S	
Rhynchoscolex simplex	K04:40, K04:41, K05:04	ITS, 28S, COI, 18S	Rostrum, no paratomy
Stenostomum arevaloi	K04:53, K04:80, K05:60	ITS, 28S, COI, 18S	Three pairs of refractile bodies, tail, epidermal inclusions
'*Stenostomum* island'	K04:81, K0:90, K04:93, K04:94	ITS, 28S, COI	Found only on Öland and Gotland
Stenostomum leucops	K04:18, K04:75, K04:88, K04:29, K04:51, K04:63, K04:85, K04:87, K05:26, K05:37, K05:51, K05:55; GenBank	ITS, 28S, COI	Refractile bodies composed of small granules
Stenostomum sphagnetorum	K04:01, K04:104, K05:07	28S, COI, 18S	Round refractile bodies
Suomina turgida 1	K04:28, K04:43	ITS, 18S	Ciliated preoral swelling; found in Jämtland
Suomina turgida 2	K04:08, K04:22, K04:32, K05:24	ITS, 18S	Ciliated preoral swelling; found in Bohuslän

supported group in the analysis of the combined data and in all single-gene datasets except 18S rDNA. The small ciliated pits are characteristic for this species. The sister group of '*A*. smallpit' is '*A*. bigmouth'. These two groups have very different morphologies; there can be no doubt that they are separate species.

The undescribed '*Anokkostenostomum* longpit' (Fig. 4O) is represented by four specimens forming a highly supported group in the combined analysis and in all single-marker datasets except 18S rDNA. '*Anokkostenostomum* longpit' is characterized by the long ciliated pits in the anterior part of the worm.

'*Anokkostenostomum* mountain' (Fig. 4Q) is a small worm with dense ciliation represented in our material by a single specimen collected in a small stream at the slope of Storsnasen mountain in Jämtland. It is the sister group to *A. bryophilum* and *A. grabbskogense* in the analyses of the combined dataset and the ITS-5.8S partition. It is tentatively regarded as a distinct species due to the limited material.

The 'island group', '*Stenostomum* island' (Fig. 4H–K) consists of four specimens that are distinct morphologically. All four were found exclusively on the Baltic islands of Öland and Gotland. The group is well supported in the analysis of the combined dataset and in all single-partition analyses except the 18S rDNA dataset. One specimen (Fig. 4K) is very similar to *S. leucops* (Fig. 4E), another (Fig. 4J) is similar to *S. sphagnetorum* (Fig. 4F). Three of the four specimens have refractile bodies. Despite these morphological differences the molecular data strongly indicate that the specimens are more closely related to each other than to other catenulids. It is unclear how many species are represented by these four terminals. Denser sampling on the Baltic islands is needed. We tentatively regard the 'island group' as a geographically isolated catenulid clade that is undergoing rapid speciation.

Nomenclatural acts

Synonymization of *Suomina* and emended diagnosis of *Catenula*

The genera in Catenulida are distinct and can be identified easily based on morphological characteristics. The Catenulidae in our dataset are classified in the genera *Catenula* and *Suomina*. In our analysis of the combined dataset *Catenula* is not monophyletic, *C. macrura* being the sister group to our *Suomina* terminals. Since the *C. macrura–Suomina* clade is the sister taxon of *C. lemnae*, *S. turgida* is transferred to the genus *Catenula* here. As *S. turgida* is the type species of *Suomina*, the two other species so far included in the latter genus are transferred to *Catenula* as well (Table 6). However, the latter two species have not been studied by us; thus their systematic position must be considered as preliminary and requiring further study.

Genus *Catenula* Dugès, 1832

Anortha Leidy, 1851

Suomina Zacharias, 1902, syn. n.

Diagnosis: Catenulidae with cephalic region delimited posteriorly by ciliated preoral groove, with or without statocyst. Preoral swelling with ciliated furrows absent or present. With or without epidermal rhabdoids.

Table 6. List of species for which a change in genus assignment is proposed

Species	Nomenclatural act
Catenula evelinae (Marcus, 1945)	Comb. n.
Catenula sawayai (Marcus, 1945)	Comb. n.
Catenula turgida (Zacharias, 1902)	Comb. n.
Stenostomum anops Nuttycombe & Waters, 1938	Original combination reinstated
Stenostomum brevipharyngium Kepner & Carter, 1931	Original combination reinstated
Stenostomum corderoi Marcus, 1945	Original combination reinstated
Stenostomum gigerium Kepner & Carter, 1931	Original combination reinstated
Stenostomum grabbskogense (Luther 1960)	Original combination reinstated
Stenostomum karlingi (Luther 1960)	Original combination reinstated
Stenostomum mandibulatum Kepner & Carter, 1931	Original combination reinstated
Stenostomum membranosum Kepner & Carter, 1931	Original combination reinstated
Stenostomum predatorium Kepner & Carter, 1931	Original combination reinstated
Stenostomum pseudoacetabulum Nuttycombe & Waters, 1938	Original combination reinstated
Stenostomum romane Kolasa, 1981	Original combination reinstated
Stenostomum saliens Kepner & Carter, 1931	Original combination reinstated
Stenostomum tuberculosum Nuttycombe & Waters, 1938	Original combination reinstated
Stenostomum ventronephrium Nuttycombe, 1932	Original combination reinstated

Synonymization of *Anokkostenostomum* and emended diagnosis of *Stenostomum*

Recently a new genus, *Anokkostenostomum*, was introduced for former *Stenostomum* species that lack refractile bodies in the anterior part of the animal (Noreña et al. 2005). In our analyses *Anokkostenostomum* is non-monophyletic, being nested among *Stenostomum* species (Fig. 1). We therefore synonymize *Anokkostenostomum* and recombine the 14 species names in it with the genus name *Stenostomum* (Table 6). The new species collected by us and initially identified as *Anokkostenostomum* are also assigned to *Stenostomum*.

Genus *Stenostomum* O. Schmidt, 1848

Weldonia Martin, 1908

Ependytes Picken, 1937

Anokkostenostomum Noreña, Damborenea & Brusa, 2005, syn. n.

Diagnosis: Stenostomidae without either statocyst or preoral ciliated furrow. Paired ciliated pits associated with the anterior cerebral lobes. Light-refracting bodies present in variable number and arrangement, or absent. Epithelial rhabdoids and excretophores present in some species. Zooid chains formed through paratomy. Sexually mature stages rare.

General remarks

Inferred branch lengths in the combined dataset are much shorter in Stenostomidae than in Catenulidae–Retronectidae. This is correlated with morphological variation: there are more species-identification problems in our stenostomids. Catenulids are direct developers without any known effective dispersal stages. Reproduction is normally through paratomy; sexually mature specimens are rarely encountered. Therefore, the dispersal ability of catenulids is probably low, and we are likely to observe various stages of speciation and population differentiation.

The 28S rDNA and ITS-5.8S partitions were the single-gene datasets that identified most of the species groups. Both deeper nodes and species groups were resolved with the 28S rDNA, whereas the ITS-5.8S partition did not resolve the deeper nodes. In several cases the ITS-5.8S partition was the only one that revealed internal structure within what we consider species-level taxa. The ITS-5.8S partition seems the ideal candidate for studying species delimitation and population divergence in Catenulida. The COI partition performed nearly as well, but was more problematic to amplify and sequence. Clearly, 18S rDNA is not variable enough to detect species-level clades in Catenulida.

We chose to recognize as separate species clades that were supported by more than one of the four molecular markers and were supported by morphological characters. This is a relatively pragmatic and instrumental 'definition' of a species, and it is possible that our nominal species can be subdivided further, depending on which species concept is used. Here we wish to avoid introducing species names for units that cannot be identified with morphological characters, and we hope that future workers will exercise similar restraint.

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